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Human Adult Spermatogonial Stem Cells for Autologous Cardiovascular Tissue Engineering

INAUGURAL-DISSERTATION
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vorgelegt von
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1 Abstract

Background

This work investigates the availability of pluripotent adult spermatogonial stem cells in human testis tissue. These cells may play an important role in tissue engineering and regenerative medicine applications and serve as autologous adult stem cells, offering an alternative to embryonic stem cells and thereby overcoming the ethical controversy of these cells.

To date testis derived pluripotent adult stem cells have only been isolated from mouse and rat tissue (1, 3, 5, 6, 10, 11, 13, 14, 17-21, 23, 25). These investigations report an isolation of the cells with the aid of collagenase, followed by purification with magnetically activated cell sorting most efficiently for CD 90 and a cell characterisation with fluorescent activated cell sorting with a number of antigens including CD 49f and CD 29. Cultures of the cell populations were established most efficiently with media including growth factors such as GDNF, bFGF, as well as EGF and LIF. The authors described that a maintenance of the cells was possible, however not an expansion of the generated tissues.

Materials and methods

Here, human tissues were obtained by subcapsular orchiectomy or testis biopsies, before they underwent enzymatic digestion with collagenase, followed by sorting of the cells with Magnetically Activated Cell Sorting (MACS) for the following antibodies.

Cells were categorized as CD 90 negative and positive, of which latter ones were subsequently cultured in the first investigations in different culture media, including EBM-2, DMEM with 10% FBS or α MEM with IGF, bFGF and GDNF, in order to search for the best compatible culture medium for testis derived human adult spermatogonial stem cells.

Furthermore CD 90 positive cells were differentiated into muscle cells, endothelial cells, adipocytes and osteoblasts, placing them into the designated differentiating media respectively.

For proof of pluripotent like characteristics, the CD 90 positive cells were analysed by Fluorescent Activated Cell Sorting (FACS), investigating the expression of CD 49f (Integrin α6) and CD 29 (Integrin β1).

The differentiated cell lines were microscopically analysed by immunofluorescence using Thrombomodulin, von-Willebrandt Factor and Phalloidin primary antibodies for endothelial cells and α Actinin, EH-Myomesin and Phalloidin primary antibodies for myocyte cells or simple dye staining including Oil Red O for osteoblasts and Alizarin Red for adipocytes.

Results

The FACS analysis revealed that the isolated cells which were previously sorted by MACS for CD 90 surface antibodies, expressed CD 29 (Integrin β1) in co-expression of CD 49f (Integrin α6).

The isolated cells could be differentiated into the destined cell lines such as muscle cells, endothelial cells, adipocytes and osteoblasts, using the appropriate medium.

This could be confirmed by immunofluorescent microscopy, which showed positive expression of von-Willebrandt Factor, Thrombomodulin and Phalloidin in endothelial cells as well as α Actinin, EH-Myomesin and Phalloidin in myocytes and Oil Red O for Osteoblasts and Alizarin Red for adipocytes.

Discussion

As these cells have not been isolated before from human testis tissue, materials and methods in order to isolate, sort, differentiate and characterize these cells had to be established in this work.

One of the barriers was the isolation of cells from the solid testis tissue. More difficulties were experienced to digest the tissue, the older the patients were.

This was paralleled by the differentiation potential of the cells probably decreasing with age of the patients, as fewer cells could be differentiated into the desired tissue, comparing old specimens to younger ones.

It should be investigated, whether, if at all this procedure could go into clinical application, the isolation of the testicular derived stem cells should be conducted at a young age by diagnostic biopsies.

It could not clearly be shown, which antibody was the most effective for MACS sorting. In mouse tissue, CD 90 was used as the standard marker (3, 11, 13, 14), however in this study CD 29 (Integrin β1) and CD 49f (Integrin α6) were shown to be comparable.

The lack of an unambiguous cell marker also leads to an anaculture, from which it is not certain to derive stem cells, as it would in single cell clones.

Cells could be differentiated successfully, however efficient expansion of tissue, as it would be required in tissue engineering, did not occur.

After all, this data suggests the potential of a population of pluripotent like stem cells in human adult testicular tissue and might be a further step towards cell based autologous regenerative medicine and tissue engineering.

2 Introduction

2.1 Tissue engineering

Tissue engineering comprises the use of living cells for the development of new tissues for improvement or replacement of defective tissues and organs, for example blood vessels, heart valves or bladder tissue.

This biotechnological field becomes especially useful in surgery involving the implantation of extrinsic or artificial implants, where the most important factors for success of the implant are the functionality and adaptability of the implant, as well as the immunological compatibility.

Taken cardiac valve replacement surgery as an example, options given are biological tissues (e.g. porcine heart valve) or artificial valves. Biological valves do not induce drastic rejection reactions against foreign bodies, e.g. activation of blood clotting, as artificial valves might do and therefore do not need anticoagulation therapy compared with artificial valves. However biological valves degenerate and commonly need replacement after ten years. This is just one example to show that organ replacement as well as transplant surgery bear many risks and are not yet sufficiently satisfactory.

Tissue engineering could overcome these limitations. With tissues developed from autologous cells from patients, the problem of immunological reactions could be depleted. There would be no shortage in donor organs for transplant patients and the quality of human tissue would be more efficient than that of e.g. porcine tissues. Engineering steps include the isolation of stem cells by biopsies from tissues containing stem cells, purification and proliferation of the isolated cells and finally seeding these cells on a graft in order to grow them into the desired shape and structure.

2.2 Adult spermatogonial stem cells

Stem cells line up in the very first front of cellular differentiation. All cells in grown tissues are to their speciality differentiated stem cells.
Pluripotent stem cells are capable of differentiating into cells of all three germ layers, i.e. forming endoderm, mesoderm and ectoderm. They are rarely found in human adult tissue and if so, only very few in number.
Multipotent progenitor cells in contrast are only capable of differentiating into different cell types belonging to just one cell lineage, for example different types of blood cells belonging to the mesodermal blood cell lineage. These cells are amply found in bone marrow, blood or skin tissue as well as other organs.

Pluripotent stem cells have been proven to be present in adult mouse testis tissue (1, 3, 5, 6, 10, 11, 13, 14, 17-21, 23, 25). These cells have not yet committed to differentiation, and still posses the capability to develop into any kind of tissue; in their foreseen manner in testis into spermatogonial, later developing into gametes and further more into spermatozoa and for that reason are called adult spermatogonial stem cells.

2.3 Adult spermatogonial stem cells in tissue engineering

So far, one of the concepts of tissue engineering includes the isolation of embryonic stem cells and to induce them to differentiate into the wanted type of tissue.
However the controversial discussion about embryonically derived stem cells is present. Ethical problems such as materialisation of an unborn human prohibit or limit the use of embryonic stem cells.

Adult spermatogonial stem cells could mark a critical change in this issue, because being adult stem cells, there is no reason for ethical compunction compared to embryonic stem cells.
A further issue is that allogenic embryonic stem cells own certain immunological characteristics which could cause rejection when transplanted. With spermatogonial stem cells being autologously applied, i.e. donor and recipient is the same person, immunological rejection can be ruled out.
Furthermore, spermatogonial stem cells seem to possess a great advantage because they can easily be biopsized with no greater harm to the testicular tissue, cryoconserved and in the case of need be engineered for elderly male patients, when it comes to tissue or organ replacements, such as heart valve disease.

For example these cells could be used in tissue engineering, generating endothelial cells from them, from which blood vessels could be constructed. Or when being explanted prior to a spermatogenesis harmful treatment, these cells could be re-implanted after the treatment and thereby induce new spermatogenesis in the male testis.

2.4 Study outline

In this study the possibility of isolation of pluripotent like stem cells from human adult testicular tissue was examined, followed by the investigation of the feasibility of the testicular tissue digestion. The sorting of the cells was analyzed by Magnetically Activated Cell Sorting (MACS) before the cells were characterized for their antigens by Fluorescent Activated Cell Sorting (FACS). A further step of this research was the assay for culture of the presumable adult spermatogonial stem cells and the determination of their differentiation potential by differentiating the obtained cells into cells of the three germ layers.

Hypothesis

- Adult human spermatogonial stem cells, exhibiting pluripotent like character, can be isolated from adult human testicular tissue
- Pluripotent like adult human spermatogonial stem cells can be sorted by MACS using CD 90
- Pluripotent like adult human spermatogonial stem cells can be characterized by FACS using CD 29 and CD 49f
- Pluripotent like adult human spermatogonial stem cells can be cultured and expanded
- Pluripotent like adult human spermatogonial stem cells can be differentiated into cells of all the three germ layers, i.e. endoderm, mesoderm and ectoderm.

3 Background: Experiences with adult spermatogonial stem cells isolated from mouse or rat testis tissue

Up to date, the successful isolation of spermatogonial stem cells had only been shown for samples collected from mouse and rat tissue (1, 3, 5, 6, 10, 11, 13, 14, 17-21, 23, 25). The different steps are summarized as follows.

3.1 Isolation of spermatogonial stem cells from mouse tissue

Testes were surgically removed from mice, the tunica was removed and the tissue was transferred into tubes containing digestion medium, consisting of 0.5 mg/ml collagenase type IV and 0.25 mg/ml trypsin. Testes were digested for 15 minutes at 33°C to dissociate tubules. (1, 3, 6, 11, 13, 17-21, 23)

3.2 Purification of the spermatogonial stem cell population with MACS sorting

Purification was achieved with magnetically activated cell sorting with different antigens, most efficiently CD 90. (3, 11, 13, 14)

3.3 FACS characterization of spermatogonial stem cells

The isolated cells have been characterized by their common surface antigen expressions being investigated by fluorescent activated cell sorting (1, 3, 4, 9-11, 13, 14).

The most promising antigens used were CD90+, CD34-, CD9+, CD49f+, CD29+, CD24+, CD117-, MHC1-.

3.4 Culture of murine spermatogonial stem cells

The authors of multiple papers describe, that the maintenance, but only modest expansion of the spermatogonial stem cell population was possible (17, 19, 21, 23).

Furthermore it was reported that the probable pluripotent stem cell like cell population could be cultured most efficiently when the medium was supplemented with glial cell line derived neurotrophic factor (GDNF), while maintaining their stem cell like character. This effect could be observed at concentration of GDNF at 100ng/ml, but not for only 10ng/ml (23).

Others found, that the breakthrough for maintaining the cells in culture came, when they included GFRα1 and bFGF to the culture medium containing GDNF, achieving expansions of the cell culture in the short term. When only two of these growth factors were added, only a slight increase in cell proliferation was observed, however it was increased when using a combination of all these three additives (19). This was confirmed by other groups, using concentration of 1ng/ml of bFGF and 40ng/ml of human GDNF (17). Another interesting finding was that cells could be best maintained when the medium was serum free (17). This was confirmed by another group, even stating, that FBS was detrimental to the expansion of spermatogonial stem cells, causing a reduction of up to 60% of the activity of these cells (19). One group using 10% FBS in combination with α MEM medium could report an expansion of the pluripotent stem cells, and presented, that growth factors like LIF, bFGF and IGF-1 appeared to have a more negative than positive effect on spermatogonial stem cell maintenance in vitro (17). This statement seems arguable, as some researchers achieved spermatogonial stem cell proliferation, using glial cell line-derived neurotrophic factor (GDNF), epidermal growth factor (EGF), basic fibroblast growth factor (bFGF) and leukaemia inhibitory factor (LIF) (19, 21, 23, 25).

4 Materials and methods

4.1 Patients

Samples were obtained from eight patients in total, of which all were on surgical treatment for prostate cancer (n=7) or testicular torsion (n=1). The patients' age ranged from 21 to 83 years, in average 58.9 years. All patients were informed about the study and the voluntary donation of testicular tissue and signed an informed consent. The study had been approved by the ethics committee of the UniversitätsSpital Zürich, Switzerland (Ref.Nr. StV. 32-2008).

4.2 Isolation of the tissue

Donor testis tissue was collected by subcapsular orchiectomy in patients with prostate cancer and by testis biopsy in patients with testicular torsion.

After the surgical explantation, the tissue was collected and transported in DMEM (Dulbecco's Modified Eagle Medium, Invitrogen, Paisley, UK) medium without any supplements.

Isolation of the cells from the tissue

The testicular tissue in one piece, was placed on the bottom of a Petri dish and frazzled by simple mechanical dissociation with scalpel and forceps, in order to pulpify the tissue. The product then underwent enzymatic digestion with collagenase A (Roche Diagnostics GmbH, Mannheim, Germany), 20 mg/ml DMEM medium (Dulbecco's Modified Eagle Medium, Invitrogen, Paisley, UK) (serum free), for 60 minutes at 37°C. To stop the enzymatic reaction, DMEM medium containing fetal bovine serum (Gibson, Nitrogen, Paisley, UK) was added, followed by resuspension of the mixture.

Whenever the connective tissue could not well enough be digested, a 500µm mash was used, to separate the dissociated cells from the rest of the connective tissue. The product was then centrifuged for 10 minutes at 1500 rpm (at 20°C) before the supernatant was discarded and the remaining cell pellet used, for further investigation.

4.3 MACS – Magnetically Activated Cell Sorting

Labelling cells with antibodies:

The cell pellet was resuspended with 90 µl PBS (Phosphate buffered saline, Kantonsapotheke Zürich, Switzerland) buffer. 10 µl of FITC (Fluorescein isothiocyanate) labelled antibodies for CD 90 (Abcam, Cambridge, UK) were added, following incubation for 10 minutes at 4°C.

In another approach 10 µl of FITC (Fluorescein isothiocyanate) labelled antibodies for HLA-ABC (Immunotools GmbH, Friesoythe, Germany) were added, following incubation for 10 minutes at 4°C.

2 ml of PBS buffer were supplemented and the mixture was centrifuged for 10 minutes at 1500 rpm. Afterwards the supernatant was discarded.

Then the cell pellet was resuspended with 90 µl buffer and additionally 10 µl of anti-FITC labelled magnetic beads (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany). Incubation then followed for 15 minutes at 4°C.

For another time, the mixture was diluted with 2 ml PBS buffer and then centrifuged for 10 minutes at 1500 rpm. After the final discard of the supernatant, cells were resuspended in 500 µl buffer.

A MACS syringe was fixed onto a provided magnet (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany), attaching to the according metal plate. A falcon tube, 15 ml, was placed under the syringe, for collection of the cells, before 500 µl of buffer were placed into the MACS syringe, the liquid dripping into the provided tube below. Once it had run through completely, the 500 µl cell suspension prepared earlier was given into the syringe. After the syringe was emptied and the liquid collected in the same tube below, it was washed three times with 500 µl buffer each. The collected cells in the tube were then negatively labelled cells.

For the collection of the positive cells, the syringe was taken out of the magnet, filled up with 1000 µl PBS buffer, closed with the provided plunger and emptied into a separate tube.

4.4 FACS – Fluorescent Activated Cell Sorting

At first, cells were counted, using a Neubauser slide.

The cells in a suspension were then split into polystyrene tubes, one each for a different antibody to be investigated, at least 100.000 cells for each. In this case, CD 29 and CD 49f surface markers were to be investigated and with that, IgG1 and IgG2a respectively as positive controls.

The tubes were centrifuged for 10 minutes at 1500 rpm, following discard of the supernatant and a resuspension with 2 ml PBS buffer each and another centrifugation for 10 minutes at 1500 rpm.

After discarding the supernatant, 1 µl of FITC labelled antibody (Immunotools GmbH, Friesoythe, Germany) was added to each tube for every 100.000 cells – provide a 1 µl of antibody dilution in 100 µl PBS buffer.

Incubation was done for 30 minutes at 4°C. The mixture was then resuspended with 2 ml PBS buffer, following centrifugation for 10 minutes at 1500 rpm and the supernatant was discarded. 1 ml PBS was added again to the remaining cell pellet and resuspended, following storage of the tubes on ice, until FACS analysis was conducted with FACScan (Becton Dickinson Biosciences, San Jose, USA).

4.5 Media

4.5.1 Culture medium:

For the investigation which culture medium was most suitable for culture of human adult testicular derived stem cells, the cells were isolated and sorted, according to the MACS protocol described previously and then cultured in different culture media including different combinations of supplements.

The obtained cells were cultured in the following different media in 12 well plates with each well containing 1.5 ml medium and renewal of the medium every three days. The cultures were passaged when the cells became confluent (using Trypsin EDTA, Gibco, Invitrogen, Paisley, UK).

The cells were inspected under a phase contrast microscope (Carl Zeiss AG, Feldbach, Germany).

The following media were tested:

(a)

DMEM[*1]	
FBS[*2]	10ml/100ml

[*1]Dulbecco's Modified Eagle Medium, Invitrogen, Paisley, UK

[*2]Fetal Bovine Serum, Gibco, Invitrogen, Paisley, UK

(b)

αMEM[*1]	
IGF[*2]	1.5µg/100ml
bFGF[*3]	0.1µg/100ml
GDNF[*4]	0.4µg/100ml

[*1]Non Essential Media, Gibco, Invitrogen, Paisley, UK

[*2]Insulin-like growth factor, Immunotools GmbH, Friesoythe, Germany

[*3]Basic fibroblast growth factor, Immunotools GmbH, Germany

[*4]Glial derived neurotrophic factor, Immunotools GmbH, Germany

(c)

EBM-2[*1]	
FBS[*2]	2ml/100ml
FGF[*3]	0.1ml/100ml
EGF[*4]	0.1ml/100ml
GM[*5]	0.1ml/100ml
IGF[*6]	0.1ml/100ml

[*1]Endothelial basal medium, Lonza, Walkerville, USA

Supplements provided by the supplier in combination with the medium

[*2]Fetal Bovine Serum, Lonza, Walkerville, USA

[*3]Fibroblast growth factor, Lonza, Walkerville, USA

[*4]Endothelial growth factor, Lonza, Walkerville, USA

[*5]Gentamycin, Lonza, Walkerville, USA

[*6]Insulin-like growth factor, Lonza, Walkerville, USA

4.5.2 Differentiation medium:

The differentiation potential of the CD 90 positive cells, was investigated, using differentiation media and followed by staining after four to six weeks of culture.

Endothelial cell differentiation (26):

EBM-2	
FBS	20ml/100ml

Osteoblast cell differentiation (26, 27, 30):

α MEM	
FBS	10ml/100ml
Ascorbic Acid[*1]	50 µg/ml
b-glycerol-2-phosphate[*2]	10 mM
Dexamethasone[*3]	10 µM

[*1,2,3]Sigma Aldrich, Germany

Adipocyte cell differentiation (26, 27):

α MEM	
FBS	10ml/100ml
Dexamethasone[*1]	1 M
Insulin[*2]	10 µg/ml
Indomethacin[*3]	120µM
IBMX for induction No IBMX for culture	0.5mM for 3 days

[*1,2,3]Sigma Aldrich, Germany

Neuroblast cell differentiation (33):

DMEM	
FBS	2ml/100ml
DMSO[*1]	1.25ml/100ml

[*1]Dimethylsulfoxid, Sigma Aldrich, Germany

and

α MEM	
FBS	20ml/100ml
β-Mercaptoethanol	1 mM/ml
bFGF	5 ng/ml

Myocyte cell differentiation (27):

DMEM	
Horse serum[*1]	10ml/100ml
Azazytidine[*2] for induction	1ml/100ml for 2 days

[*1]PAN Biotech GmbH, Aidenbach, Germany

[*2]Sigma Aldrich, Germany

4.6 Staining

In order to verify the differentiation process, the cells were stained for their specific cell line, after four to six weeks of culture as described above.

4.6.1 Adipocytes

Cells were washed in their culture dishes with PBS and later fixed in 4% formalin PBS solution for 30 minutes. After the formalin was removed, the cells were washed twice with distilled water and once with 60% Isopropanol in PBS, following application of the stain onto the cells: 50 mg Oil Red O (E. Merck (Schweiz) AG, Dietikon, Switzerland) were dissolved in 10 ml 100% Isopropanol. Then a working solution with a ratio of 3:2 stock solution:distilled water was prepared, from which was given onto the cells in their dishes. After 10 minutes, the cells were rinsed once with 60% Isopropanol and twice with distilled water, before being visualized under the microscope (Carl Zeiss AG, Feldbach, Germany) for detection of adipocytes.

4.6.2 Osteoblasts

The cells were washed in their culture dishes with PBS, following fixation with 4% formalin PBS solution for 30 minutes. After removal of the fixative, the dish was washed three times with distilled water, before Alizarin Red S stain (Fluka Chemie AG, Buchs, Switzerland) was applied onto the cells – 2% in water, pH 4.2 with 10% ammonium hydroxide. Incubation time was 10 minutes, the stain was removed and the cells washed three times with distilled water and once with PBS. The cells then could be observed under the microscope, for detection of osteoblasts.

4.6.3 Endothelial cells

The cultured cells were washed with PBS and fixed in 4% PFA/PBS solution for 10 minutes at room temperature. After removal of the fixative, the cells were washed another time in PBS, following a block with 0.1 M Glycine (Sigma Aldrich, Germany) in PBS for 5 minutes, before continuing with permeabilization in 0.2% Triton X100/PBS (Sigma Aldrich, Germany) for 10 minutes. After washing with PBS, a block with 5% goat serum in 1% BSA/PBS was applied for 30 minutes. Incubation of the primary antibodies was done with Thrombomodulin (Dako, Zug, Switzerland), von-Willebrandt Factor (Sigma Aldrich, Germany) and Phalloidin (Alexa Fluor 488 Phalloidin, Invitrogen, Carlsbad, USA), each diluted in 5% goat serum in 1% BSA/PBS at concentrations of 1:50, 1:200 and 1:100 respectively, for 1 hour at room temperature. The cells were then washed and the secondary antibodies FITC (Fluorescein isothiocyanate conjugated, Sigma Aldrich, Germany) and Cy3 (Cyanine dye, Jackson Immuno Research, West Grove, USA) were applied, in 1:100 and 1:200 dilutions respectively, incubating for 45 minutes at room temperature. Cells were then rinsed with PBS three times in their dishes and finally embedded in Lisbeth`s medium, containing 0.1 M Tris-HCL (pH 9.5) / glycerol (3:7) with 50 mg/ml n-propyl gallate as anti fading reagent (Donation from I. Agarkova, University Hospital Zurich, Switzerland). Finally the cells were inspected under the immunofluorescent microscope (Leica Microsystems AG, Herbrugg, Switzerland).

(Part of the dish with the endothelial cells was separated and stained with the dyes used for myocytes, as negative control.)

4.6.4 Myocytes

The cultured cells were washed with PBS and fixed in 4% PFA/PBS solution for 10 minutes at room temperature. After removal of the fixative, the cells were washed another time in PBS, following a block with 0.1 M Glycine in PBS for 5 minutes, before continuing with permeabilization in 0.2% Triton X100/PBS for 10 minutes. After washing with PBS, a block with 5% goat serum in 1% BSA/PBS is applied for 30 minutes. Incubation of the primary antibodies was done with α Actinin (Sigma Aldrich, Germany), EH-Myomesin (Donation from I. Agarkova, University Hospital Zurich, Switzerland) and Phalloidin (Alexa Fluor 488 Phalloidin, Invitrogen, Carlsbad, USA), each diluted in 5% goat serum in 1% BSA/PBS at concentrations of 1:500, 1:1000 and 1:100 respectively, for 1 hour at room temperature. The cells were then washed and the secondary antibodies FITC and Cy3 were applied, in 1:100 and 1:200 dilutions respectively, incubating for 45 minutes at room temperature. Cells were then rinsed with PBS three times in their dishes and finally embedded in Lisbeth`s medium, containing 0.1 M Tris-HCL (pH 9.5) / glycerol (3:7) with 50 mg/ml n-propyl gallate as anti fading reagent. Finally the cells were inspected under the immunofluorescent microscope.

(Part of the dish with the myocyte cells was separated and stained with the dyes used for endothelial cells, as negative control.)

5 Results

5.1 Isolation

In the first attempts 0.5 mg/ml collagenase were used to digest the testis tissue obtained through orchiectomy, showing no dissociation of the tissue. In further trials the concentration of the digestive solution was increased to 2 mg/ml collagenase, resulting in scattered dissociation of single cells, which blocked up when used in the syringes for the following MACS sorting. At last a final working concentration of 20 mg/ml collagenase caused the connective tissue to be digested for the most part and there was no clot formation after centrifugation of the cells suspension. The so obtained cells were free from other debris, large in number and could be used in MACS syringes for sorting (Figure 1).

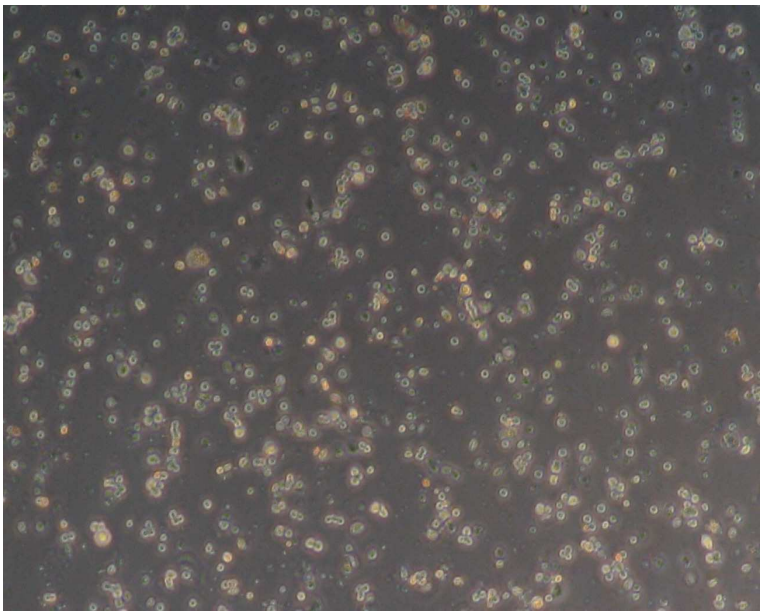


Figure 1: Cells after tissue digestion with collagenase

5.2 MACS – Magnetically Activated Cell Sorting

The numbers of cells obtained through the MACS for CD 90 positive antibody are shown in Figure 2, ranging from approx 300.000 to approx. 2.000.000 per testis tissue sample, in average approx. 1.170.000 cells per testis tissue sample (n=5), irrespective of the size of the sample, i.e. ectomy or biopsy.

Patient #	Patients' Age (Years)	No. of cells isolated after CD 90 MACS
6	83	440.000
4	77	300.000
5	58	1.640.000
3	55	1.480.000
7	21	1.980.000

Figure 2: Table showing the number of cells isolated in selected patients

Further antibodies such as CD 49f and CD 29 were explored with magnetically activated cell sorting. The results for CD 49f showed an isolation number ranging from 200.000 to 1.500.000, in average 810.000 cells per testis tissue sample (n=5) and for CD 29 from 350.000 to 1.000.000, in average 670.000 cells per testis tissue sample (n=5).

5.3 FACS – Fluorescent Activated Cell Sorting

CD 49f analysis:

In the side scatter diagram of the FACS analysis it could be seen that two cell populations were found in the cell suspension (Figure 3). The more dense and greater of these two was suspected to be the population containing CD 49f positive cells (Figure 4).

In another subpopulation (P4 in figure 4) the amount of parent cells is 20.7% (27.1 % CD 49f - 6.4 % control). Measured against the total number of cells, 12.6% (17.1 % CD 49f – 4.5 % control) of the total cells may be assumed to be CD 49f positive.

CD 29 analysis:

In the side scatter diagram of the FACS analysis it could be seen that two cell populations were found in the cell suspension (Figure 5). The more dense and greater of these two was suspected to be the population containing CD 29 positive cells (Figure 6).

In another subpopulation (P4 in figure 6) the amount of parent cells is 7.7% (21.1 % CD 29 – 13.4 % control). Measured against the total number of cells, 4.7% (13.6 % CD 29 – 8.9 % control) of the total cells may be assumed to be CD 29 positive.

The average of all cell samples was as follows

CD 49f positive selection	Ø Parent 18.6%
	Ø Total 10.9%
CD 29 positive selection	Ø Parent 5.7%
	Ø Total 2.7%

FACS CD 49f

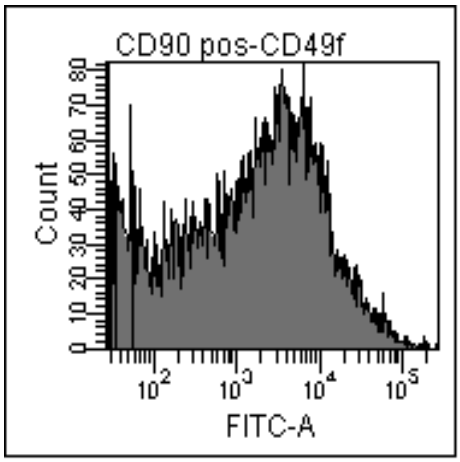


Figure 3: Cell counts of all events

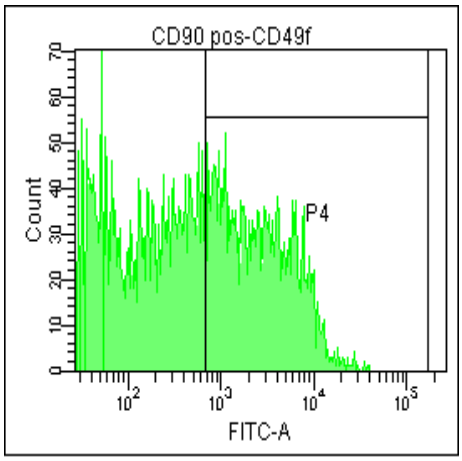
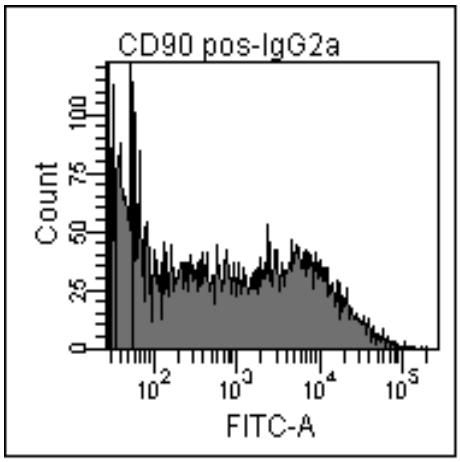
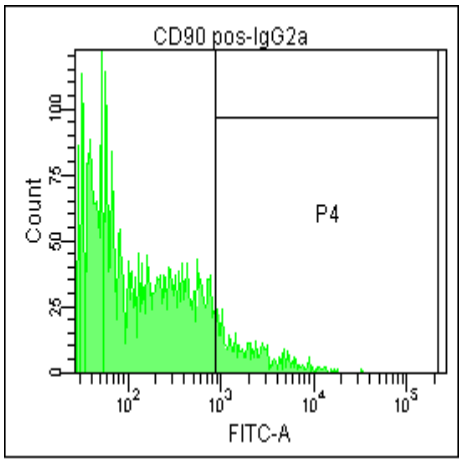


Figure 4: Cell counts of the greater subpopulation extracted from all events

FACS IgG2a Control



Cell counts of all events



Cell counts of the greater subpopulation extracted from all events

FACS CD 29

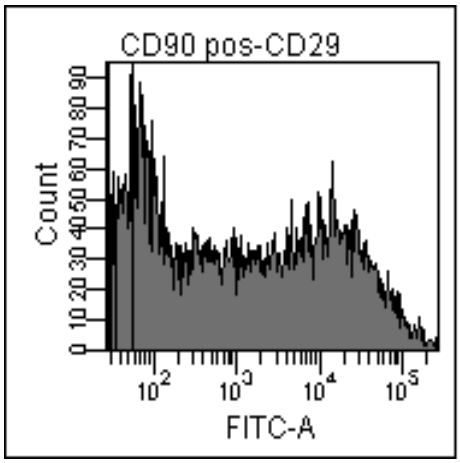


Figure 5: Cell counts of all events

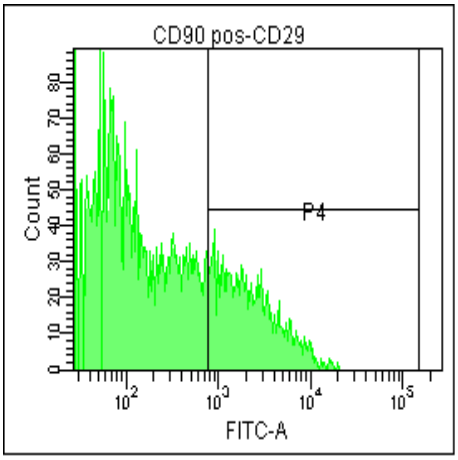
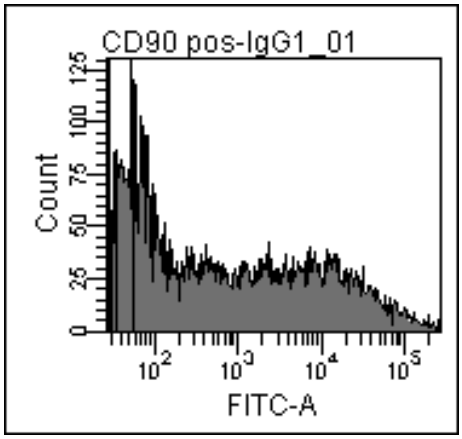
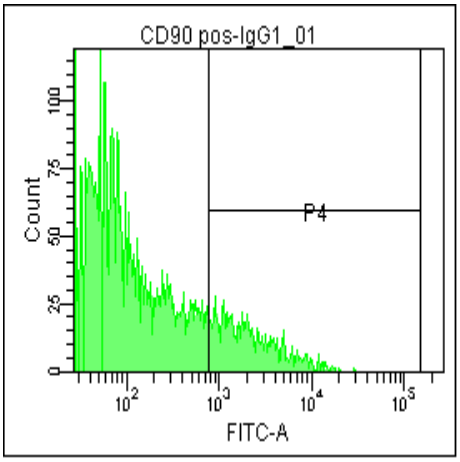


Figure 6: Cell counts of the greater subpopulation extracted from all events

FACS IgG1 Control



Cell counts of all events



Cell counts of the greater subpopulation extracted from all events

5.4 Culture

In all samples investigated the number of cells critically diminished until absolute loss of all cells in less than two weeks, when the cells were kept in α MEM medium containing IGF 1.5 µg/100ml, GM 0.1 ml/100ml, bFGF 0.1 µg/100ml, GDNF 0.4 µg/100ml. The cell number was in average reduced from initially 250.000 at the time of setting up the culture, to only 20.000 later one week, a drop of 92% (Figure 7 and 8).

The DMEM containing media, supplemented only with GM 0.1 ml/100ml and FBS 10 ml/100ml showed a reduction from in average initially 250.000 at the time of setting up the culture, to as little as 88.000 cells, counted after one week. This resembles a decrease of 65% (Figure 7 and 8).

When EBM-2 medium in combination with FBS 2ml/100ml, FGF 0.1 ml/100ml, EGF 0.1 ml/100ml, GM 0.1 ml/100ml, IGF 0.1 ml/100ml was used for maintenance of the adult testicular derived stem cells, the loss was found to be 43% in the first week, from initially 250.000 cells to 144.000 cells in average (Figure 7, 8 and 9).

Different test series included the use of DMEM medium with no supplemented growth factor. This condition showed similar results.
Another attempt was the use of EBM-2 medium in combination with the growth factors used with the α MEM medium; IGF, bFGF and GDNF. Furthermore the effects of the growth factor used in combination with EBM-2 (FBS, FGF, EGF, IGF), DMEM (FBS) and α MEM (IGF, bFGF, GDNF) were investigated by combinations at different concentration than those already mentioned. However, in none of these alternative trials could the cells be maintained for longer than two weeks nor be expanded.

Although the mentioned loss was observed during the first two weeks, the cells exposed to the EBM-2 medium proliferated very effectively after this time and could be maintained for over five weeks. The proliferation rate was high, and the number of cells cell doubled every seven days.

Patient #	Patients' Age (Years)	No. of cells isolated after CD 90 MACS	Culture Medium	No. of cells put in culture	No. of cells after 7 days	No. of cells after 14 days	No. of cells after 21 days	No. of cells after 28 days
6	83	440.000	EBM	100.000	65.000	40.000	75.000	130.000
			DMEM	100.000	40.000	15.000	5.000	-
			α MEM	100.000	5.000	-	-	-
5	58	1.640.000	EBM	400.000	220.000	150.000	240.000	400.000
			DMEM	400.000	140.000	33.000	-	-
			α MEM	400.000	40.000	-	-	-
3	55	1.480.000	EBM	300.000	130.000	150.000	200.000	345.000
			DMEM	300.000	90.000	27.000	-	-
			α MEM	300.000	27.000	3.000	-	-
7	21	1.980.000	EBM	200.000	160.000	135.000	190.000	460.000
			DMEM	200.000	80.000	20.000	4.000	-
			α MEM	200.000	7.000	-	-	-

Figure 7: Table showing cell numbers of selected cultures in the first 4 weeks

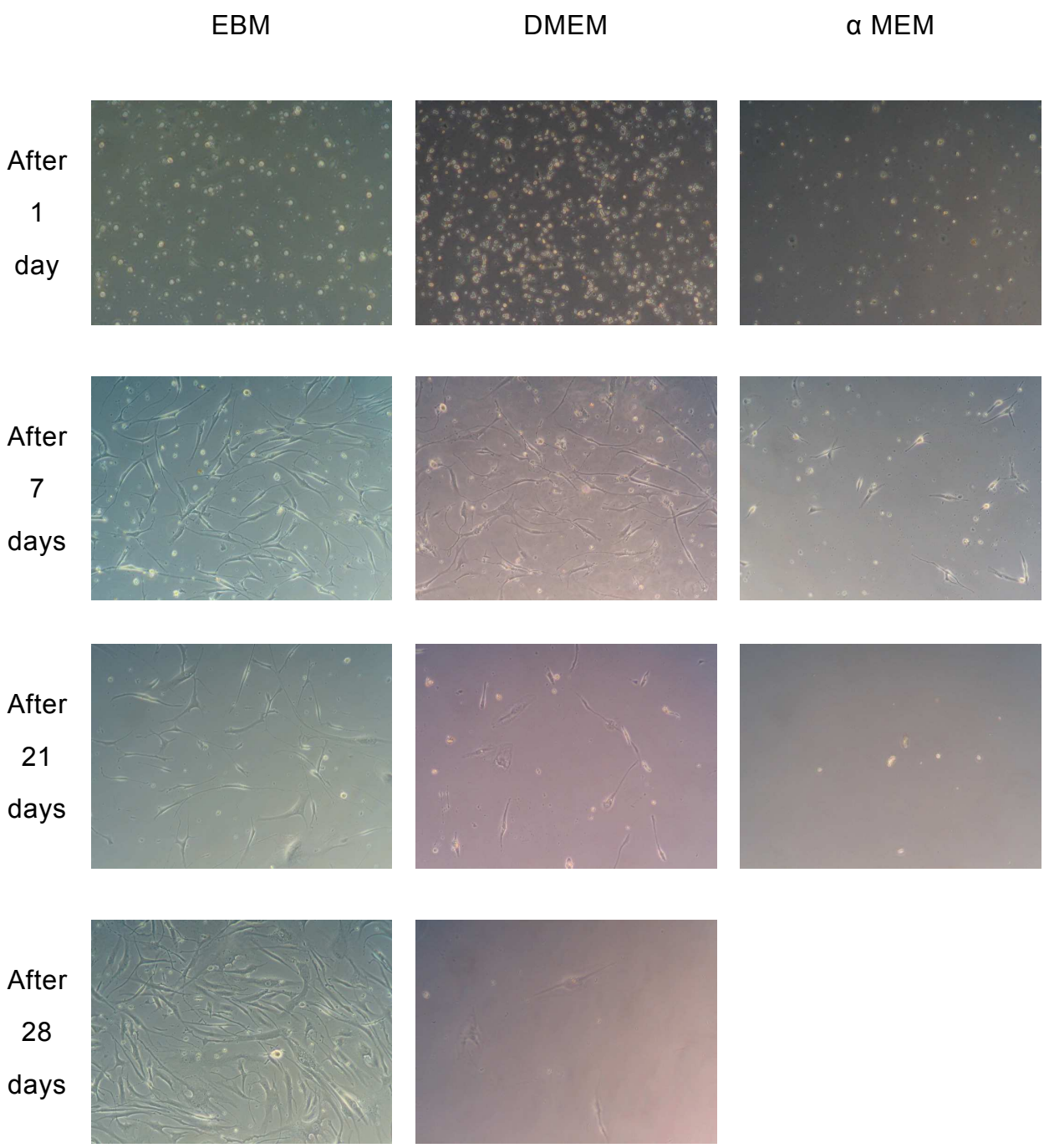


Figure 8: Cells cultured in EBM, DMEM and α MEM after 1, 7, 21 and 28 days

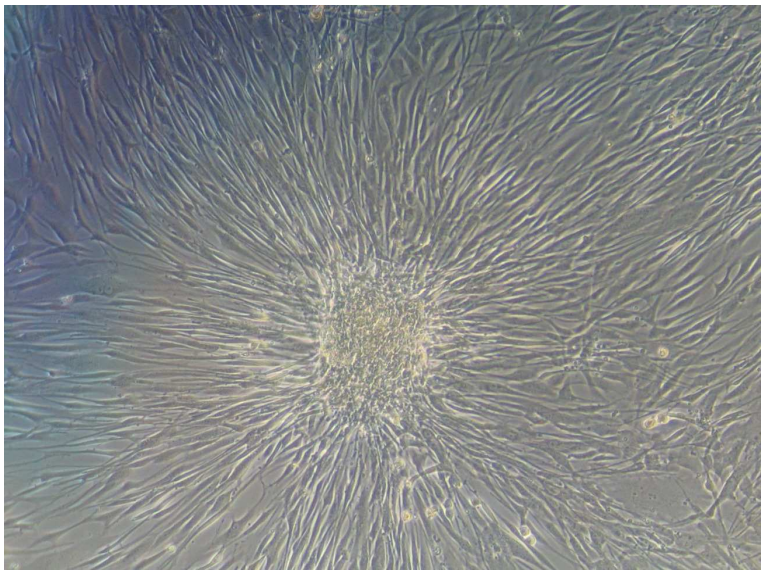


Figure 9: MACS CD 90 positive cells in EBM-2 medium after 19 days

Two non-differentiated cell lines were frozen after the process of isolation and again thawed and differentiated later. The potential for differentiation was no different to that of cells before cryoconservation.

However, cells that had already been differentiated and afterwards cryoconserved, showed only very little ability to multiply after defrosting and died within 10 days.

5.5 Differentiation and staining

In one set of cell lines (n=5), the cells were exposed to the differentiation media, after one week of culture in EBM-2 medium, which was shown to be the most adequate condition for CD 90 positive testicular derived stem cells to be maintained. This was done, because the cells after isolation were only very little in number (approx. 980.000 cells). A control was conducted directly after isolation, with a fewer number of cells. It could thus be observed, that in an earlier point of differentiation, the potential of the cells might seem more effective, as the cells kept in the EBM-2 medium acquainted little morphological change. However, the number directly after isolation was very small and the cells did not proliferate when kept in the differentiating media, they only altered phenotypically. The cells on which the differentiation was induced after one week post isolation, differentiated into their destined cell type and their number was higher than those differentiated directly after isolation.

In another set of cell lines (n=3), all of the isolated cells were induced to differentiate directly after isolation and magnetic sorting for CD 90 positive cells. The isolated cell number was very high (approx. 1.390.000 cells) and there was no need to expand the tissue prior to differentiation.
The cells were kept to differentiate for five weeks and then analysed by staining. The culture of the cells showed no problems and in contrast to the previously described cell line, the cells proliferated at a high proliferation rate during differentiation, making it necessary to passage the cultures every 6 days.
(Figures 10-14)

Cells after 27 days of differentiation

Figure 10: Control

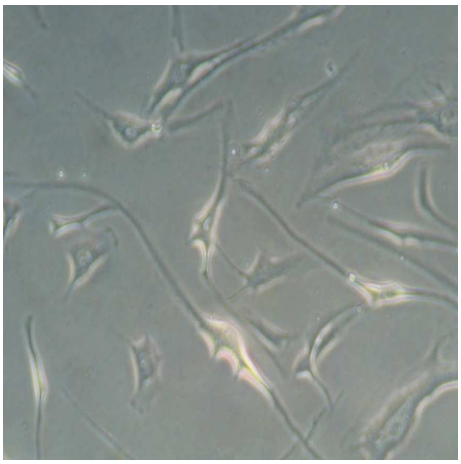


Figure 11: Myocytes

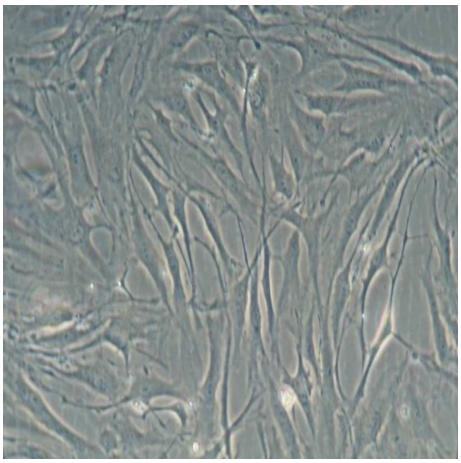


Figure 12: Osteoblasts

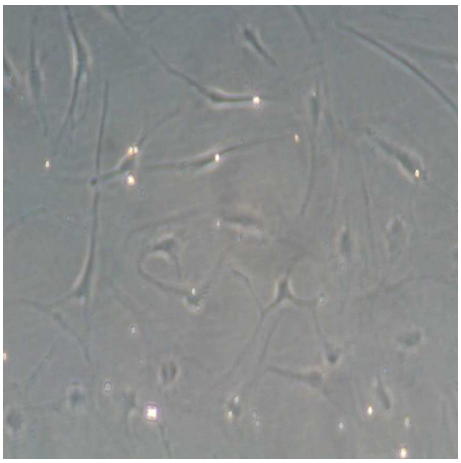


Figure 13: Adipocytes

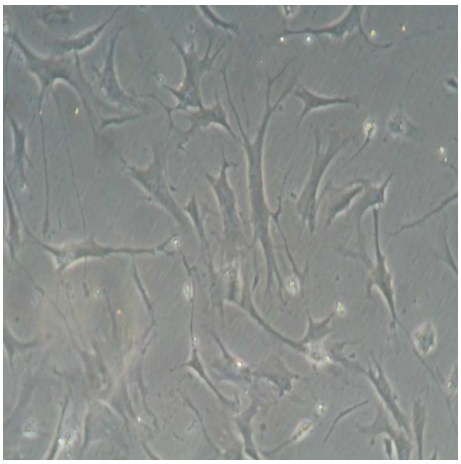
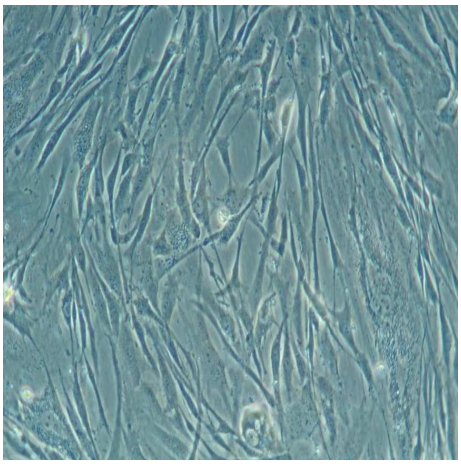


Figure 14: Endothelial



The staining of the endothelial differentiating cell line showed an expression of Thrombomodulin, von-Willebrandt Factor and Phalloidin (Figure 16). In the immunofluorescent dyes of myocyte differentiating cell lines, α Actinin, EH-Myomesin and Phalloidin could be demonstrated (Figure 15).
The adipocyte cell lines successfully acquired the staining of the Oil Red O dye, while the osteoblast cell lines took on the Alizarin Red S stain, both being investigated under a phase contrast microscope.

It needs to be mentioned, that the two earlier mentioned neurocyte differentiation media which had been used, provoked cell death after a few days.

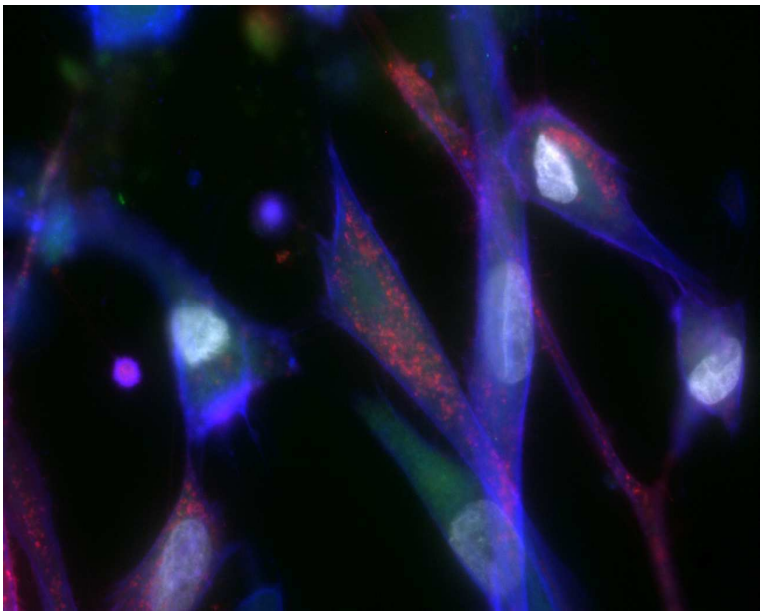


Figure 15: Myocytes. Green: EH-Myomesin, Red: α Actinin, Blue: Phalloidin

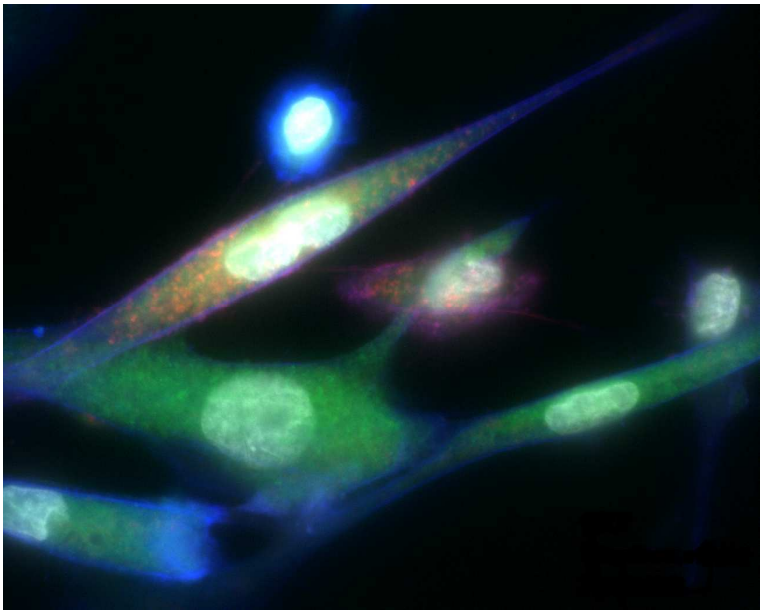


Figure 16: Endothelial cells. Green: von-Willebrandt Factor, Red: Thrombomodulin, Blue: Phalloidin

6. Discussion

6.1 Discussion of the results

6.1.1 Isolation

In the first attempts it was difficult to digest the testis tissue obtained by orchiectomy. Previous reports from mouse tissue digestion described to have used 0.5 mg/ml collagenase for dissociation of the connective tissue (1, 3, 6, 11, 13, 17-21, 23).

As this was not potent enough to generate enzymatic digestion of the tissue, 2 mg/ml collagenase were used in the following isolations of the human testis tissue. With this the connective tissue was not sufficiently digested for the subsequent MACS sorting. Therefore only a small amount of purified cells could be isolated and sorted by the MACS.

Small progresses were achieved by additionally exposing the cell suspension to trypsin 0.05% for 15 minutes. This made the reunion of the dissociated tissue in centrifugation less critical.

Another option explored was to filter the tissue in order to separate tough connective tissue, from smaller cells. For this a 500 µm mash had been used, formed like a cone and fixed on a funnel. The filtered cell suspension could smoothly be used in the syringes for the following MACS analysis.

The breakthrough was achieved, when 20 mg/ml collagenase was used. The connective tissue could for the most part be digested and there was no clot formation after centrifugation of the cells suspension.

6.1.2 MACS – Magnetically Activated Cell Sorting

Provided the cell separation from the testis tissue was accomplished successfully, the MACS process itself showed no greater difficulties.

In the first attempts the magnetically labelled antibody HLA-ABC was used, in reference to the hypothesis, that spermatogonial stem cells are part of the very rarely found HLA-ABC negative cells. However, this thesis could not be confirmed, as the amount of HLA-ABC negative cells and the HLA-ABC positive cells were almost identical. However, it should be well noted, that it was found, that the lack of HLA-ABC antibody on spermatogonial stem cells could be confirmed by FACS analysis (data not shown).

Further antibodies such as CD 49f and CD 29 had been explored with magnetically activated cell sorting. However, the results could not prove to be superior to those of CD 90 (revealed through cell count). In the following sortings, this was set as the default antibody used for cell sorting. The antibodies CD 49f and CD 29 were subsequently used for the fluorescent analysis of the sorted cells (FACS).

Also a combination of HLA-ABC negative and CD 49f, CD 29 and CD 24 positive cells was investigated in MACS analysis. The verification with FACS analysis however could not prove the sorting to have been successful.

The cells obtained through the MACS for CD 90 positive antibody were very different in number, ranging from approx 300.000 to approx. 2.000.000 per testis tissue sample, irrespective of the size of the sample, i.e. ectomy or biopsy.

The patients` age ranged from 21 to 83 and as age was rising, the concentration of obtained cells decreased dramatically (Figure 2).

6.1.3 FACS – Fluorescent Activated Cell Sorting

The main focus was put upon the investigation of CD 90 positive cells, being sorted out prior to FACS by MACS. These cells were analysed for surface markers Integrin α6 (CD 29) and Integrin β1 (CD 49f), using IgG1 and IgG2A as controls respectively. The results proved that CD 49f and CD 29, both markers of pluripotent like stem cells, are co-expressed in the investigated cells and thus suggest that these cells exhibit stem cell like surface characteristics.

6.1.4 Culture

The investigations on which culture medium would be the most suitable to culture human adult derived spermatogonial stem cells showed, that the best results for maintenance and expansion of the CD 90 positive cells was to use EBM-2 culture medium with the corresponding growth factor supplements provided by the manufacturer.

It should be mentioned, that although there was a significant loss observed during the first two weeks, the cells exposed to the EBM-2 medium proliferated very effectively after this time. Being cultured in the EBM-2 medium, the cells could be maintained for over five weeks.

6.1.5 Differentiation and staining

The differentiation media used showed a successful differentiation of the spermatogonial adult stem cells into endothelial (Figure 16) and myocytes (Figure 13) cell line, as well as of the osteoblast (Figure 14) and adipocyte (Figure 15) differentiation. However not for neurocyte cells.

The staining showed positive results for the endothelial differentiating cell line, as well as the myocytes in the immunofluorescent dyes. The adipocyte and osteoblast cell lines resembled positive results, being investigated under a phase contrast microscope after simple dye staining.

6.2 Discussion of the subject matter

It could successfully be shown, that from human adult testicular tissue, cells can be isolated, which exhibit pluripotent stem cell like behaviour, i.e. are able to be differentiated into cell lines of different germ layers. It could be suggested, which culture supplements play a role in expansion of the cells, while maintaining their stem cell like potential. We could show that MACS selection with specific labelled antibodies is effective for first selection of the desired stem cells.

Recent studies have primarily investigated the properties of adult spermatogonial stem cells in mouse and rat species and not human derived cells (1, 3, 5, 6, 10, 11, 13, 14, 17-21, 23, 25). Thus a limitation of this study is the lack of an established human control cell, to which this study could be referred to, especially because the definition of the term pluripotency incorporates different criteria in mouse and human stem cells.

It was clearly experienced, that the potential of separation of the testis tissue and the differentiation of the isolated cells was very diverse, depending on the patients` age. The best results were acquired with a twenty-one year old patient, where no difficulties in the enzymatic digestion of the connective tissue were experienced and the differentiation potential of the stem cells was enormously stronger, than that of other samples.

Related to this, the source of the tissue samples represents a debatable aspect. The obtained tissue sample were taken from two origins; orchiectomy in patients with metastasizing prostate cancer, or testis biopsies from patients with testicular torsion. It could be observed, that the latter, although much

smaller in size, provided a higher concentration of pluripotent cells, as well as a better quality in terms of potential to differentiate.
It should be discussed, whether donation of cells for tissue engineering purpose could become a standard procedure of diagnostics in patients with testicular torsion, thus providing a potentially valuable source of these cells. However the feasibility for these biopsies should be further investigated.

The question remains, whether expansion of the freshly isolated and not yet differentiated cells results in higher cell number, than cells being differentiated right after isolation and later being expanded. Again cells samples from older patients showed slightly different behaviour to those of younger age groups. The cells from elder patients showed a lower rate of multiplication after differentiation after a short period of culture than before, where as the younger cells expanded quicker after the process of differentiation.

Further exploration on what surface markers for magnetically activated cell sorting serve the most efficient results might seem needful. Antibodies such as CD 90, CD 49f and CD 29 all seem promising candidates, but lack quantitative characterisation of expression on human adult testis derived stem cells.
A simultaneous isolation had been tried, but resulted in no exploitable data. A successive selection of the markers, following each other in multiple MACS passages could be shown to be a promising approach.

A heterogeneous culture of cells could be differentiated into cells of different germinal layers, thus suggesting a pluripotent potential among the cells. However to be certain of the potential of one specific cell to exhibit pluripotency, a single cell clone has to be obtained, cultured and differentiated.
As a further step to achieve separation and culture of a single cell clone of pluripotent human adult testicular stem cells, attachment to specifically coated culture dishes (e.g. vimentin) or a broader selection of distinct surface markers, as indicated above could be tried.

Endothelial staining proved to be positive for Thrombomodulin and Phalloidin, however only weakly for von-Willebrandt Factor. This suggests, that von-Willebrandt Factor is only being expressed in a later stage of cell development. In future studies it should be analyzed with different stains, at what stage of differentiation of the cells, which cell-typical cell organelles are being formed, e.g. in myocytes. A promising method could be transmission electron microscopy.

It has to be considered, that due to possible genetic change occurring while the cells are being cultured, the cells may turn into uncontrolled proliferation stages. One of the most critical points of controlling the potential and the time of differentiation of the stem cells, is the composition of the culture medium. Therefore strict monitoring of genetic alterations is a prerequisite to the re-implantation of the human adult pluripotent stem cells in clinical practice.

6.3 Outlook

The main goal of this study was to isolate human pluripotent stem cells from adult – non-embryonic - tissue and to investigate their usability for tissue engineering cell based therapies, presenting a key step to the resolution of the ethical problem when working with allogenic embryonic stem cells. Recent studies have successfully demonstrated, that this can be conducted on adult testis of mice and rats (1, 3, 5, 6, 10, 11, 13, 14, 17-21, 23, 25).

It could be shown, that in patients from which only a testicular biopsy had been gained, the young age made it possible, that from this minute mass real tissue engineered textures could be generated. Taken this into account, it shall be investigated, whether this could be sufficient enough even for elderly patients, with no therapeutic need of orchiectomy.

Also the concentration of cells gained through MACS sorting still serves the chance to increase. If the proper combination of antibodies has been declared, the chances for developing well functioning differentiated cells are very good.

Starting from the isolation of the tissue, the enzymatic digestion should be improved, especially with the fibrous connective tissue in elderly patients. Many cells degenerate in the processing with enzymes and are mechanically harmed through the magnetic sorting cells sorting process and passaging cultures. The next step should be isolation with lower rates of centrifugation and to omit trypsin for passaging, using only tools of cell-specific attachment for subculturing.

The most critical improvements which need to be done are higher concentrations in isolating and sorting the cells from the testis tissue, procedures of resecting only ultimately small testis tissue samples from biopsies and a greater rate of expansion of the cells while maintaining their pluripotent potential to differentiate.

After all, this data suggests the potential of a population of so named pluripotent like stem cells in human adult testicular tissue and might be a crucial piece to the solution to cell based autologous regenerative medicine and tissue engineering.

At last it should be mentioned, that recently a group of researcher around Skutella et al. published the discovery of pluripotent human adult testicular derived stem cells (34). Their investigations were based on MACS isolation of the cells and evidence of pluripotency through formation of teratoma, cell line differentiation into all three germ layers and demonstration of specific marker-genes.
This paper has now been strongly discussed among stem cell researchers, as the analytic proof does not seem substantiated and fully convincing to term the declared all-rounder cells “truly pluripotent”.

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I dedicate this work to the advancement of sciences and hope to contribute to the indispensable progress in medical treatment.

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9 Curriculum Vitae

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